

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	H. U. BERGMAYER(ED.) 'Methods of Enzymatic Analysis' 1985 , VCH VERLAGSGESELLSCHAFT MBH. , WEINHEIM, DE 3rd. edition, Vol. VII. see page 110 - page 117 ---	1,7-10, 12, 15-17, 22,27, 29,31, 36-38
Y	H. U. BERGMAYER (ED.) 'Methods of Enzymatic Analysis' 1985 , VCH VERLAGSGESELLSCHAFT MBH , WEINHEIM, DE 3rd. edition, Vol. VII see page 357 - page 364 ---	1,7-10, 15-17, 21,27, 29,30, 36-38
A	H. U. BERGMAYER (ED.) 'Methods of Enzymatic Analysis' 1985 , VCH VERLAGSGESELLSCHAFT MBH , WEINHEIM, DE 3rd. edition, Vol. VII, pages 403-409 ---	
A	CLINICAL CHEMISTRY. vol. 31, no. 4, April 1985, WINSTON US pages 624 - 628 H. REFSUM ET AL. 'Radioenzymatic determination of homocysteine in plasma and urine' cited in the application -----	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9300138
SA 69139

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 21/04/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A-2549853	01-02-85	JP-C- 1446904	30-06-88
		JP-A- 60030679	16-02-85
		JP-B- 62054470	16-11-87
		US-A- 4609626	02-09-86
EP-A-0070033	19-01-83	JP-C- 1449138	11-07-88
		JP-A- 58011857	22-01-83
		JP-B- 62057220	30-11-87
		CA-A- 1177750	13-11-84
		US-A- 4478934	23-10-84

PATENT COOPERATION TREATY

PCT

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

09/857,433

Applicant's or agent's file reference 44.772334/001	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/GB01/01615	International filing date (day/month/year) 10/04/2001	Priority date (day/month/year) 10/04/2000
International Patent Classification (IPC) or national classification and IPC G01N33/48		
Applicant AXIS-SHIELD PLC et al.		

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TECH CENTER 1800/2900

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 8 sheets, including this cover sheet.
 - ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 07/11/2001	Date of completion of this report 03.07.2002
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Klee, B Telephone No. +49 89 2399 2675



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB01/01615

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17):*):

Description, pages:

1-30 as originally filed

Claims, pages:

31,32 as originally filed

33,34 as received on 13/06/2002 with letter of 13/06/2002

Drawings, sheets:

1/3-3/3 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB01/01615

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 7-11, 21-23, 26, 27.

because:

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
 - ☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 7-11, 21-23, 26, 27 are so unclear that no meaningful opinion could be formed (*specify*):
see separate sheet
 - ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
 - ☐ no international search report has been established for the said claims Nos. .
2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:
- ☐ the written form has not been furnished or does not comply with the standard.
 - ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/GB01/01615

- ☒ restricted the claims.
- ☒ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.
2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
- ☐ complied with.
- ☐ not complied with for the following reasons:
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
- ☐ all parts.
- ☒ the parts relating to claims Nos. 1, 4, 5, 6, 12-20, 24, 25.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1, 4-6, 12-20, 24, 25
	No:	Claims
Inventive step (IS)	Yes:	Claims 1, 4-6, 12-20, 24, 25
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1, 4-6, 12-20, 24, 25
	No:	Claims

2. Citations and explanations
see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

See item 3

Re Item IV

Lack of unity of invention

1. The separate groups of invention are:

1. Claims 1, 20 and 19 (dependent on claim 1) and claims 21-27 (dependent on claim 20).
2. Claim 2.
3. Claims 4, 5, 6 and dependent claims 12-19 .

1.1 First group of inventions

With respect to claims 1

D1 discloses an assay for homocysteine which comprises contacting a biological fluid sample with a reducing agent (column 7, lines 19-25, column 18, line 25 and column 30, line 43-49) and subsequently with homocysteine desulphurase (column 5, lines 26-36, column 9, line 55-56) from which the subject matter of claim 1 differs in that said sample is contacted with an agent which binds, oxidizes or depotentiates said reducing agent after being contacted with said homocysteine desulphurase.

The technical effect of contacting the sample with an agent which binds, oxidizes or depotentiates said reducing agent after being contacted with said homocysteine desulphurase is to avoid side reactions of DTT.

Claim 20 encompasses the same specific technical feature. Therefore claims 1, 20 and dependent claims 19, 21-27 form the first group of inventions.

1.2 Second group of inventions

With respect to claim 2

D1 discloses an homocysteine assay which comprises contacting a biological fluid with a liquid reagent containing a homocysteine converting enzyme (column 5, lines 4-25), from which the subject matter of claim 1 differs in that said reagent is produced by adding an aqueous liquid to a lyophilisate containing said enzyme and a cryo/lyoprotectant, characterized in that said lyophilisate is substantially free

of thiol-containing cryo/lyoprotectants.

The technical effect of the addition of an aqueous liquid to a lyophilisate containing said enzyme and a cryo/lyoprotectant, wherein the lyophilisate is substantially free of thiol-containing cryo/lyoprotectants is to avoid side reactions of a thiol-containing cryo/lyoprotectants.

Therefore claim 2 forms the second group of inventions.

1.3 Third group of inventions

With respect to claim 4

D1 discloses a homocysteine assay which comprises contacting a biological fluid sample with a homocysteine converting enzyme (column 5, lines 4-25), from which the subject matter of claim 4 differs in that before contact with said enzyme said sample is treated with an agent which serves to deactivate pyruvates.

The technical effect to avoid the polymerization of pyruvate in the sample.

Claims 5, 6 and dependent claims 12-19 encompass the corresponding technical feature (immobilization of the enzyme and removal of the fluid sample, with respect to claim 5; removal of pyruvate by filtration). Therefore claims 4, 5, 6 and dependent claims 12-19 form the third group of inventions.

1.4 No additional fee has been paid with regard to the second group of inventions claim 2. Therefore the examination concerns the first (claims 1, 20 and dependent claims) and third group of the inventions (claims 4, 5, 6, 12-19 and dependent claims).

1.5 As a service to the applicant the analysis of claim 3 is provided although the examination of claim 3 has not been requested:

Novelty (Art.33(2) PCT) with respect to claim 3:

D1 discloses a homocysteine assay which comprises contacting a biological fluid sample with a liquid reagent containing homocysteine desulphurase (column 5, lines 26-36, column 9, line 55-56), wherein said liquid reagent is an aqueous liquid containing homocysteine desulphurase, a thiol-reducing agent (column 7, lines 19-25), and a proteinaceous stabilizer or non-proteinaceous stabilizer (implicitly disclosed, since buffers used in protein chemistry usually comprise stabilizers) therefore the subject matter of claim 3 is not new in view of D1.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/GB01/01615

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

2. References cited:

D1: US-A-5 998 191

D2: WO 00 00821 A

D3: WO 98 59242 A

D4: EP-A-0 726 322

3. Clarity (Art.6 PCT)

3.1 Claim 20 is not clear and concise ("optionally", "e.g.).

3.2 Claims 7-11 are not clear and concise.

3.3 Claims 21-23 and 26 are not concise (features A, B, C, D) and claim 27 is not legible.

4. Inventive step (Art.33(3)PCT)

4.1 With respect to claims 1 and 20 (first group of inventions)

Document D1, which is considered to represent the most relevant state of the art, discloses an assay for homocysteine which comprises contacting a biological fluid sample with a reducing agent (column 7, lines 19-25, column 18, line 25 and column 30, line 43-49) and subsequently with homocysteine desulphurase (column 5, lines 26-36, column 9, line 55-56) from which the subject matter of claim 1 differs in that said sample is contacted with an agent which binds, oxidizes or depotentiates said reducing agent after being contacted with said homocysteine desulphurase.

The problem to be solved by the present invention may therefore be regarded as to avoid side reactions with DTT.

None of the documents cited indicates that side reactions of DTT can be avoided by contacting the sample with an agent which binds, oxidizes or depotentiates the reducing agent after being contacted with said homocysteine desulphurase. In contrast D1 (column 18, line 23-25) favours the use of DTT .

Therefore the subject matter of claim 1 is inventive in view of D1-D4.

The same applies to claims 20 which encompasses the same specific technical feature (an agent which binds, oxidizes or depotentiates said reducing agent) and to claim 19 dependent on claim 1 .

4.2 With respect to claims 4, 5, 6 and claims dependent thereon

Document D1, which is considered to represent the most relevant state of the art, discloses a homocysteine assay which comprises contacting a biological fluid sample with a homocysteine converting enzyme (column 5, lines 4-25), from which the subject matter of claim 4 differs in that before contact with said enzyme said sample is treated with an agent which serves to deactivate pyruvates.

The problem to be solved by the present invention may therefore be regarded as to avoid the polymerization of pyruvate in the sample.

None of the documents cited provides a method to deactivate pyruvates before a contact of a sample with the enzyme. Therefore the subject matter of claim 4 is inventive in view of D1-D4.

The same applies to claims 5, 6 and claims 12-19 dependent thereon.

With respect to claim 5: None of the documents cited provides a method to immobilize the enzyme and contact the immobilized enzyme under such time and conditions to allow the homocysteine in the sample to bind to the enzyme and then to remove the fluid sample from the assay to avoid polymerization of the pyruvates.

With respect to claim 6: None of the documents cited provides a method to remove pyruvate by filtration before the contact of a sample with the enzyme. Therefore the subject matter of claims 5, 6 and claims 12-19 dependent thereon is inventive in view of D1-D4.

- 33 -

15. An assay as claimed in claim 4 wherein the agent which serves to deactivate pyruvates is pyruvate carboxylase.
16. An assay as claimed in claim 4 wherein the agent which serves to deactivate pyruvates is pyruvate oxidase.
17. An assay as claimed in claim 4 wherein the agent which serves to deactivate pyruvates is lactate dehydrogenase.
18. An assay as claimed in claim 6 wherein the sample is filtered with a 30 kD exclusion filter.
19. An assay as claimed in any one of claims 1 to 18 wherein said homocysteine converting enzyme is HDS and wherein a NAD^+/NADH cycling reaction is used to generate a coloured compound the concentration of which may be correlated to the concentration of homocysteine in the initial biological fluid sample.
20. A kit for a homocysteine assay, said kit comprising:
 - homocysteine desulphurase;
 - a homocyst(e)ine standard;
 - a reducing agent;
 - an agent which binds, oxidizes or depotentiates the reducing agent;
 - optionally one or more further reagents capable of converting the homocysteine conversion product of homocysteine desulphurase into a detectable analyte;
 - optionally a pyruvate deactivating agent;
 - optionally a filter for removing; and
 - optionally a filter capable of removing red blood cells from blood.

21. A kit as defined in claim 20 wherein the homocysteine desulphurase is in a lyophilized form, the lyophilisate being substantially free of thiol-containing cryo/lyoprotectants.

22. A kit as defined in claim 20 wherein the homocysteine desulphurase is in a aqueous liquid form further containing a dithiol reducing agent and a proteinaceous or non-proteinaceous stabilizer.

23. A kit as defined in claim 20 wherein the homocyst(e)ine standard is a plurality of standards containing Hcy or homocysteine at a plurality of known concentrations.

24. A kit as defined in claim 20 wherein the reducing agent is dithiothreitol, dithioerythiol, TCEP or methyl iodide.

25. A kit as defined in claim 20 wherein the agent which binds, oxidizes or depotentiates the reducing agent is a maleimide.

26. A kit as defined in claim 20 wherein the pyruvate deactivating agent is hydrazine, acetoacetate decarboxylase, pyruvate carboxylase, hydrogen peroxide or pyruvate dehydrogenase.

27. A kit as defined in claim 20 wherein the filter for removing pyruvate, if present, is a molecular sieve.

(19) World Intellectual Property Organization
International Bureau



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0008784.1 **10 April 2000 (10.04.2000)** **GB**

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **HOMOCYSTEINE ASSAY**

(57) Abstract: The present invention provides an improved method of assessing/quantifying the amount of homocysteine in a body fluid sample via an enzymatic assay which comprises reducing background signal by treatment with one of the following: a reducing agent, a pyruvate deactivating agent, heat treatment, or by lyophilising or immobilizing the homocysteine converting enzyme.

WO 01/77670 A2

Homocysteine Assay

The present invention relates to improvements in and relating to enzymatic assays for homocysteine in biological fluid samples.

Elevated blood plasma homocysteine levels can be correlated to risk of cardiovascular disease, e.g. coronary heart disease, coronary artery disease, cerebrovascular disease, and peripheral vascular disorders. Indeed elevated homocysteine levels are thought to be a better predictor of cardiovascular disease than elevated cholesterol levels. Generally plasma levels of 15 μ M and below are considered healthy (see for example New England Journal of Medicine 1997, 337: 230).

Accordingly there is a need for reliable methods for determining homocysteine levels in patients.

Direct determination of homocysteine has thus far proved to be complicated, e.g. because it has not been found possible to raise an antibody against homocysteine (HCy) which is not cross-reactive with other substances present in biological samples.

However, in WO 93/15520 (Axis) and WO 98/07872 (Glasgow) for example, homocysteine assays are described which involve enzymatic conversion of homocysteine and determination of homocysteine levels by determination of a homocysteine conversion product produced by the enzyme-mediated conversion.

Such assays require the use of a reducing agent (e.g. dithiothreitol) to liberate covalently bound homocysteine and of a homocysteine converting enzyme and while they perform well there is room for improvement in terms of increasing signal to noise, e.g. by reducing background signal.

We have now surprisingly found that the performance of such enzymatic HCy assays may be improved by a

- 2 -

variety of relatively simple measures.

A first of such measures involves treatment of the biological fluid sample with a reducing agent for example a thiol (particularly a dithiol, such as dithiothreitol (DTT), dithioerythrol (DTE) or bis-(2-mercaptoethyl)sulphone), phosphine (e.g. triscarboxyethylphosphine (TCEP) or tri-n-butylphosphine), methyl iodide, thioredoxin, lipoic acid or a borohydride) to liberate covalently bound homocysteine, addition of a homocysteine converting enzyme and then treatment of the sample with an agent which neutralizes the reducing agent, e.g. one which binds to it, oxidizes it or otherwise depotentiates it, for example an organic disulphide compound or a dithiol (especially DTT or DTE) binding agent.

Viewed from one aspect therefore the invention provides an assay for homocysteine which comprises contacting a biological fluid sample with a reducing agent, especially DTT or DTE, and subsequently with homocysteine desulphurase, characterised in that said sample is contacted with an agent which binds, oxidizes or depotentiates said reducing agent, e.g. an organic disulphide or a dithiol (especially DTT or DTE) binding or depotentiating agent, for example cystamine or a maleimide, after being contacted with said homocysteine desulphurase.

Examples of suitable binding agents for thiol-based reducing agents such as DTT and DTE include maleimides, particular cyclic N-maleimides, i.e. optionally 3 and or 4-substituted, N-substituted 1-aza-2,5-dioxo-cyclopentenes, particularly compounds in which the N-substitution is to produce a bis maleimide and wherein the ring C and N substituents contain up to 25 carbons (e.g. comprising alkyl, aryl, aralkyl, aralkyl and maleimide groups). Examples of particular maleimides include N-methyl-maleimide, N-ethyl-maleimide, 1,1'-(3,3'-dimethyl-1,1'-biphenyl-4,4'-diyl)-bismaleimide,

- 3 -

1,1'-(methylene di-4,1-phenylene)bismaleimide, N-(1-phenylethyl)maleimide, 1-(2-methoxy-5-methyl phenyl)maleimide, and 2-methyl-N-phenyl-maleimide. Many of these compounds are available commercially, e.g. from Sigma Aldrich. Examples of other suitable dithiol depotentiating agents include cystamine and other compounds that reduce the reducing capacity of dithiols such as DTT, oxiranes, aziridines, aryl halides, mercurials (e.g. p-chloromercuribenzenesulphonic acid and hydroxymercuribenzoic acid), vinyl sulphones, haloacetyl compounds (e.g. iodoacetamide), 5,5-dithio-bis(2-nitro benzoic acid) (i.e. Ellman's Reagent) and disulphide exchange reagents such as pyridyl sulphides (e.g. 4,4-dipyridyl-disulphide).

The reducing agent (e.g. DTE, DTT or TCEP) is conveniently added to the biological fluid sample at a concentration of 0.5 to 5 mM, especially preferably about 1 mM. Where the reducing agent is not a dithiol, an organic disulphide rather than a dithiol binding or depotentiating agent will be used. The organic disulphide or dithiol binding or depotentiating agent is typically added to a concentration of 0.05 to 20 times the reducing agent concentration used, preferably 0.1 to 10 times, particularly 0.2 to 1.0 times, especially 0.3 to 0.6 times. Thus it will generally be added to a concentration of 0.05 to 200 mM, especially 0.2 to 15 mM, particularly 0.2 to 1.0 mM for dithiol binding agents (e.g. maleimides) and 0.2 to 200 mM, especially 1 to 100 mM, particularly 5 to 15 mM for organic disulphides (e.g. cystamine).

The biological fluid sample used in the assay of the invention is conveniently a blood or blood derived sample, e.g. plasma or serum, although other biological fluids may be used if desired. The sample is preferably cell-free, e.g. being prepared by centrifugation, filtration, or by lysis.

The assays of the invention preferably involve the

use of a second enzyme for which a product of the enzyme-mediated homocysteine conversion is a substrate. If necessary further enzymes or other systems may be used to generate a directly detectable analyte. Especially preferably the second enzyme is lactate dehydrogenase (LDH), which in the presence of nicotinamide adenine dinucleotide (NADH) converts α -ketobutyrate to α -oxo-butyrate and NAD^+ . NAD^+ can be detected by a colour generating cycling reaction as described in WO 98/07872 (Glasgow) and in the Examples below.

The neutralization or depotentiation of DTT in such Hcy assays significantly improves the assay result by reducing background; however there is still room for further improvement.

The homocysteine desulphurase enzyme used in such Hcy assays is generally unstable on storage in aqueous solution and thus are generally provided in lyophilized form, e.g. using albumin, for example bovine serum albumin (BSA) as the cryo/lyoprotectant. We have now surprisingly found that assay background may be reduced by avoiding use of BSA, e.g. by use instead of thiol-free cryo/lyoprotectants, e.g. thiol-free albumin, immunoglobulins, polyalkyleneoxides (e.g. PEG), or sugars such as trehalose and maltose.

Thus viewed from a further aspect the invention provides a homocysteine assay which comprises contacting a biological fluid sample with a liquid reagent containing a homocysteine converting enzyme, especially HDS, wherein said reagent is produced by adding an aqueous liquid to a lyophilisate containing said enzyme and a cryo/lyoprotectant, characterised in that said lyophilisate is substantially free of thiol-containing cryo/lyoprotectants.

In this aspect of the invention, the enzymes used may be enzymes as described in WO 93/15220 (Axis), WO 98/07872 (Glasgow), US-A-5985540 (Anti Cancer), US-A-

- 5 -

5885767 (Biocatalytics), US-A-5998191 (Anti Cancer), and the publications referred to therein.

The homocysteine converting enzyme is preferably one which removes or converts the thiol group in HCy. Examples of homocysteine converting enzymes include S-adenosyl homocysteine hydrolase (SAHH), homocysteinase or homocysteine desulphurase (HDS), dimethylthetin-homocysteine methyl transferase (DHMT), methionine synthetase (MS) and cystathionine β -synthetase (C β S). Preferably however the enzyme is homocysteine desulphurase (HDS), which converts homocysteine to α -ketobutyrate.

Examples of suitable cryo/lyoprotectants (otherwise referred to as bulking agents or stabilizers) include thiol-free albumin, immunoglobulins, polyalkyleneoxides (e.g. PEG), trehalose, mannitol, glucose, maltose, raffinose and stachyose. (See for example WO 97/29782). These can be used in conventional amounts in conventional lyophilization techniques.

This second aspect of the invention is especially preferably used in combination with the first aspect of the invention.

Where second or further enzymes are used in lyophilised form, these too are preferably prepared using thiol-free cryo/lyoprotectants.

Alternatively the HDS containing reagent may be provided as a liquid containing a thiol-reducing agent (e.g. DTT, DTE, TCEP, etc.), together with a proteinaceous or non-proteinaceous stabilizer, e.g. thiol-free albumin (e.g. DTT or TCEP treated albumin) or immunoglobulin (e.g. IgG, for example bovine gamma globulin), peptones or a polyalkylene oxide (such as a PEG) or a polyol such as a fatty acid ester (e.g. C₁₆₋₂₂, especially C₁₈ fatty acid) of a polyol (especially a C₆ polyol) or a polyoxyethylated derivative thereof (e.g. a Span or Tween nonionic surfactant such as Tween 20), a carbohydrate or polysaccharide. In general,

- 6 -

proteinaceous stabilizers should be used at concentrations of up to 10% wt, e.g. 0.01 to 10%, preferably 0.01 to 1%, more preferably 0.02 to 0.5%, e.g. 0.05 to 2 mg/mL.

Thus viewed from a further aspect the invention provides a homocysteine assay which comprises contacting a biological fluid sample with a liquid reagent containing homocysteine desulphurase, wherein said liquid reagent is an aqueous liquid containing homocysteine desulphurase, a thiol-reducing reagent (e.g. at 0.05 to 20 mM, especially 0.05 to 15 mM, preferably 1 to 10 mM), and a proteinaceous or non-proteinaceous stabilizer (e.g. at 0.01 to 10% wt, preferably 0.01 to 1%, more preferably 0.02 to 0.5%).

The three aspects of the invention described above serve to reduce the background signal level, i.e. the signal generated by performance of the assay in the absence of the homocysteine conversion enzyme. However again there is still room for further improvement.

It has surprisingly been found that background levels may be reduced still further by treatment of the biological fluid sample to remove pyruvates and other keto acids.

Thus viewed from a further aspect the invention provides a homocysteine assay which comprises contacting a biological fluid sample with a homocysteine converting enzyme, characterised in that before contact with said enzyme said sample is treated with an agent which serves to deactivate pyruvates, e.g. by immobilizing, binding or converting pyruvates.

Examples of agents which deactivate pyruvates include non-enzymatic agents such as thiamine, alkaline hydrogen peroxide, dichloromethyl ether, air or oxygen, or nucleophiles such as hydrazine, semicarbazide and hydroxylamines and enzymes such as pyruvate carboxylase, pyruvate oxidase, malate dehydrogenase, transaminases, acetoacetate decarboxylase, lactate dehydrogenase,

- 7 -

pyruvate decarboxylase, 2-ketobutyrate dehydrogenase, alanine transaminase, acetolactate synthase, 2-ethylmalate synthase, urocanate hydrolase, cystathionine lyase, methylalanine dehydrogenase, N5-(carboxyethyl)-ornithine synthase, methylmalonyl Co-A carboxy transferase, glutamine-pyruvate transaminase, pyridoxamine-pyruvate transaminase, serine-pyruvate transaminase, lysine-pyruvate transaminase, ATP:pyruvate 2-O-phosphotransferase, and especially alanine amino transferase and pyruvate dehydrogenase. Thiamine promotes non-enzymatic decarboxylation of pyruvates. Hydrazines serve to trap pyruvates. Pyruvate decarboxylase and the coenzyme thiamine pyrophosphate convert pyruvate to acetaldehyde and CO_2 . Alanine amino transferase, together with glutamate and pyridoxyl-5-phosphate, converts pyruvates to 2-oxo-glutarate and L-alanine, and pyruvate dehydrogenase converts pyruvates to acetyl Co-A.

If the agent used to deactivate pyruvate is hydrogen peroxide, it will be understood that the hydrogen peroxide will need to be neutralised prior to contacting the sample with said enzyme. Any suitable reagent i.e. antioxidant or enzyme may be used, but preferably the hydrogen peroxide is neutralised using catalase, which catalyzes the following reaction: $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$.

Preferably, hydrogen peroxide is added to the sample at 0.01% to 1% final concentration, preferably 0.05% to 0.5% and most preferably 0.1 to 0.3%. Catalase is added after the removal of pyruvate at 10 to 500 U/ml, preferably 50 to 400 U/ml, most preferably 80 to 300 U/ml.

Some pyruvate-converting enzymes such as malate dehydrogenase and pyruvate dehydrogenase are NAD dependent for their action and thus may be less preferred where the homocysteine assay involves a NAD dependant signal generation step.

Enzymatic pyruvate-converting agents will generally be preferred over non-enzymatic agents due to their relative specificity as a result of which any excess agent will not give rise to undesired effects later in the assay procedure. Thus for example where hydrazine is used and the homocysteine assay involves use of HDS to generate α -ketobutyrate, the α -ketobutyrate must be protected by removal of any remaining hydrazines or other nucleophiles, e.g. by addition of ketones or more preferably aldehydes (e.g. acetone or formaldehyde) in stoichiometric amounts after the pyruvate-conversion step.

Typically, the biological fluid sample will be contacted with: 0.1 to 20 mM hydrazine (especially 0.1 to 15 mM, particularly 0.5 to 10 mM); 20 to 150 μ M glutamate (especially 25 to 100 μ M, particularly 30 to 80 μ M), 1 to 100 μ M pyridoxyl-5-phosphate (especially 5 to 80 μ M, particularly 10 to 50 μ M) and 5 to 50 IU alanine amino transferase; 1 to 100 mM pyruvate dehydrogenase (especially 1 to 60 mM, particularly 5 to 50 mM) and 1 to 100 μ M Co Enzyme A (especially 5 to 80 μ M, particularly 10 to 50 μ M); or 1 to 100 mM pyruvate decarboxylase (especially 1 to 60 mM, particularly 5 to 50 mM) and 1 to 100 μ M thiamine pyrophosphate (especially 5 to 80 μ M, particularly 10 to 50 μ M). Appropriate concentrations for such pyruvate-deactivating agents can be determined by routine experimentation on pyruvate-containing biological fluid samples.

Pyruvate removal may be effected using several reagents and several treatment steps if desired. Thus such treatment may for example involve:

- i) pre-treatment of patient samples with acid (e.g. 1M HCl) followed by centrifugation and subsequent use in the assay of the supernatant. The acid will typically be added to achieve a concentration in the supernatant of 0.1 to 1M, preferably 0.2 to 0.5M,

- 9 -

especially 0.2 to 0.3M;

ii) heat-treatment, e.g. to 37 to 100°C, preferably 37 to 80°C, more preferably 40 to 60°C, of the plasma or serum;

iii) esterification of pyruvic acid, e.g. by addition of ethanol and HCl;

iv) removal of carboxylic acid groups, e.g. by addition of 0.1 to 3 mg/mL EDAC, preferably 0.1 to 2 mg/mL, especially 0.5 to 1.5 mg/mL, and Tris buffer, e.g. 0.1 mM Tris, pH 7;

v) addition of semicarbazide, e.g. to a concentration of 0.01 to 10 mM, preferably 0.1 to 5 mM, especially 0.2 to 2 mM;

vi) addition of hydroxylamine, e.g. to a concentration of 0.01 to 10 mM, preferably 0.1 to 5 mM, especially 0.2 to 2 mM.

vii) pretreatment with LDH (generally to a concentration of 10 to 40 μ g/mL, preferably 12 to 30 μ g/mL, especially 15 to 25 μ g/mL) and NADH (generally 20 to 80 μ M, preferably 45 to 55 μ M) to convert pyruvate to lactate, and heat treatment (e.g. as above preferably in the presence of nitrous acid) to remove NAD⁺;

viii) pre-treatment with β -naphthylamine (generally to a concentration of 0.1 to 10 mg/mL, preferably 0.1 to 5 mg/mL, especially 0.2 to 1 mg/mL);

ix) if required treatment to remove NAD, e.g. by heating as described above, heating in an acid environment, or exposure to ultraviolet light; and

x) pre-treatment of patient samples via exclusion filtration followed by centrifugation in order to remove pyruvate and other keto acids. Any suitable filter may be used.

Pyruvate removal may be effected using size exclusion filters, together with centrifugation. Any suitable exclusion filter may be used, e.g. 10 kD to 60 kD exclusion filter, preferably 20 to 50 kD, most preferably 30 kD. Use of exclusion filtration followed

- 10 -

by centrifugation prior to the addition of the homocysteine converting enzyme forms a preferred aspect of the invention.

Filtering the biological fluid sample through a molecular sieve in order to remove or reduce background signals in the enzymatic homocysteine assay forms a preferred aspect of the invention.

Following the deactivation of pyruvate, it forms a preferred aspect of the invention to heat the sample, e.g. to 37 to 100°C, preferably 37 to 80°C, more preferably 40 to 60°C, for 5 to 100 minutes, preferably 10 to 80 minutes, more preferably 15 to 60 minutes. It forms a further preferred aspect if the pyruvate deactivating agent used prior to heat treatment is hydrogen peroxide.

This aspect of the assay of the invention may be performed using any of the homocysteine converting enzymes mentioned in the publications referred to above, especially HDS. This aspect of the invention is preferably used together with the first, second or third aspects, especially with both the first and second or first and third aspects.

Preferably, a microtitre plate is used in any of the aspects of the invention.

Further, the use of immobilized homocysteine converting enzymes may allow the removal of pyruvate from the serum.

Thus, viewed from a further aspect the invention provides a homocysteine assay which comprises contacting a biological fluid sample with an immobilized homocysteine converting enzyme, especially HDS, wherein said biological fluid sample contacts the immobilized enzyme under such time and conditions to allow the homocysteine in the sample to bind to said enzyme, characterised in that the biological fluid sample is then removed from the assay.

In this aspect of the invention, the enzymes used

- 11 -

may be as described previously. Preferably however the enzyme is HDS.

The homocysteine-converting enzyme may be immobilized by any suitable technique well known in the art. Preferably said homocysteine converting enzyme is attached to a solid support, via any suitable linkage. As used herein "linkage" refers to any interaction between the homocysteine converting enzyme and the solid support, enabling them to be associated. Such interaction may involve physical association such as covalent binding and may also involve so-called "weak" interactions such as hydrogen bonds, Van der Waals forces and ionic interactions. Alternatively, the homocysteine converting enzymes may be provided with means for attachment to a solid support. Such means may constitute or comprise, for example, one partner of an affinity binding pair, e.g. biotin, binding to the corresponding binding partner of the affinity binding pair, i.e. streptavidin, provided on the solid support. DNA:DNA binding proteins and antibodies:antigens may also be used as alternative binding pairs.

Alternatively, the solid support may be provided with means for attachment to a homocysteine converting enzyme. Suitable means include immobilized antibodies or fragments thereof, amine binding ligands or protein binding ligands e.g. plates coated with chelating nickel which are commercially available e.g. plates coated with chelating nickel which are commercially available (Qiagen Ltd, UK). In order to create an amine binding surface the solid support may be succinimide activated microtitre plates can either be coated with succinimide, or are commercially available (Pierce Chemical Company, US).

The solid support may be any of the well known supports or matrices which are currently widely used or proposed for immobilisation, separation, etc. These may take the form of particles, sheets, dipsticks, gels,

- 12 -

filters, membranes, fibres, capillaries or microtitre strips, tubes, plates or wells etc.

Conveniently, the support may comprise glass, silica, latex or a polymeric material as for example nitrocellulose, teflon, alginate, agarose, polystyrene, latex or nylon. Preferred are materials presenting a high surface area for binding.

Preferably, a microtitre plate may be used in the method of the invention.

The attachment of the homocysteine converting enzyme to a solid support allows easy manipulation of the enzyme. Thus, the attachment to some kind of solid support can enable the separation of the homocysteine from the rest of the components in the sample, including pyruvate. This can be achieved for example by carrying out washing steps.

The attachment of the homocysteine converting enzyme to a solid support avoids the need to use the lyophilised form of the enzyme.

Preferably, the biological fluid sample is treated with a reducing agent such as DTT, DTE or TCEP prior to contact with the immobilized homocysteine converting enzyme, in order to release covalently bound homocysteine. The sample is then contacted with the immobilized enzyme under such conditions to allow homocysteine to binding to said enzyme, i.e. for 0.5 to 10 minutes, preferably 1 to 8 minutes, more preferably 1 to 5 minutes. Once the homocysteine has bound to the homocysteine converting enzyme, the solid support may be washed in order to remove the biological fluid sample. The solid support is washed using any suitable fluid, preferably a buffer solution, most preferably phosphate buffer.

After the biological fluid sample has been removed from the immobilized enzyme, the assay may be as described previously.

Preferably, the immobilized enzyme is HDS. In

- 13 -

order to facilitate the method of this aspect of the invention, the active site or substrate binding site of the enzyme have been engineered by any suitable means, for example via genetic engineering of recombinant enzyme DNA, or by chemical treatment of the homocysteine converting enzyme, in order to chemically alter the active or binding site. With regard to HDS, treatment of the immobilized enzyme with hydrazine prior to contacting the enzyme with the biological fluid sample, removes pyridoxal 5 phosphate from the active site of the enzyme. Once the biological fluid sample has been removed from the immobilized homocysteine sample, the pyridoxal 5 phosphate moiety can be reintroduced. Removal of the pyridoxal 5 phosphate moiety from the active site of the HDS enzyme allows homocysteine to bind to HDS, but no reaction can take place until the pyridoxal-5-phosphate moiety is replaced. Therefore, the engineering of the enzyme to allow binding of homocysteine, but preventing the further enzymatic reaction, is a preferred aspect of the invention. Once the pyruvate has been removed via washing, the enzyme is treated to enable the reaction to proceed.

This aspect of the invention is preferably used together with the first aspect of the invention.

Where the biological fluid on which an enzymatic homocysteine assay is carried out is serum or plasma, we have also found that background, or more precisely patient-to-patient background variation, may be reduced if the cells are removed from the sample rapidly after blood collection, preferably within 60 minutes, more preferably within 30 minutes or less (i.e. 25, 20, 15, 10, 5 minutes or less). Accordingly it is proposed that blood samples for enzymatic assay for homocysteine should be filtered cell free, e.g. through a needle equipped with a filter, within 60 minutes of collection. This forms a further aspect of the present invention.

Viewed from this aspect the invention provides an

- 14 -

enzymatic assay for homocysteine in a blood sample, characterised in that said sample is filtered cell-free (i.e. red and white blood cell free) within 60 minutes, preferably within 30 minutes, of being withdrawn from the patient.

Filtering according to this aspect of the invention may be effected by passing the blood sample through a filter, for example a filter in a syringe needle base or in a sample receiving tube, or by absorption in an absorbent web followed by compression of the wetted web to expel a cell-free fluid sample.

The background in a enzymatic homocysteine assay may be determined by performing the assay without use of the homocysteine converting enzyme.

In the assays of the invention, the homocysteine concentration may be determined in a qualitative, semi-quantitative or quantitative manner, e.g. as an absolute concentration or as an indication that concentration is above or below a threshold value or inside or outside a particular range. Total homocysteine concentration will generally be determined; however if desired treatment with a reducing agent such as DTT, DTE or TCEP may be omitted and the free homocysteine concentration may instead be determined. To allow quantitative determination, the assay will preferably be calibrated by being run with standards containing homocysteine at a known concentration or more preferably a series of known concentrations. The background signals for the various samples tested will likewise preferably be determined so that a corrected signal may be generated by subtraction of the background signal from the detected signal. Preferably sample signals, background signals and calibration signals will be detected following performance of the assay for the same period of time, i.e. after allowing signal to build up for the same period of time. For each type of assay, the optimum period of time for signal build up may be determined

- 15 -

readily by following the time-dependence of signal and background development. Such an optimum may obviously be a balance between signal value and assay duration, especially where the assay is performed on automated apparatus.

The signal determined in the assay will depend on the nature of the reaction used to generate the signal. Thus the signal will generally be a radiation (e.g. light) absorption, emission or scattering. In the examples below, the signal is the light absorption (generally measured at 550 nm) by the colored formazan compound generated from a colourless tetrazolium salt. In this particularly preferred form of the assay of the invention, the following process steps and reactions occur:

1. A blood sample is collected and separated into plasma or serum.
2. The cells from the sample are removed, preferably within 60 minutes, more preferably within 30 minutes, e.g. by centrifugation or filtration.
3. The resultant plasma or serum sample is preferably treated with a pyruvate removing agent, e.g. hydrogen peroxide.
4. Excess pyruvate removing agent is removed (i.e. excess hydrogen peroxide is removed with catalase).
5. The sample is preferably treated with a reducing agent, especially preferably dithiothreitol (DTT), dithioerythrol (DTE), or triscarboxyethylphosphine (TCEP) to release covalently bound homocysteine.
6. The sample is contacted with HDS to convert HCy to α -ketobutyrate, H_2S and ammonia.

- 16 -

7. The HDS-treated sample is preferably treated with an organic disulphide (especially where a non-sulphide reducing agent is used in step (4)), or an oxidizing agent (e.g. perchloric acid, iodate or periodate), or more preferably a DTT binding agent (e.g. a maleimide), to bind or destroy excess reducing agent.
8. The sample is contacted with NADH and lactate dehydrogenase (LDH) and incubated to convert α -ketobutyrate and NADH to α -hydroxybutyrate and NAD^+ .
10. The sample is acidified to remove excess NADH and then brought to neutral pH.
11. The sample is contacted with ethanol, a tetrazolium salt, alcohol dehydrogenase (ADH) and an oxidizing agent causing an NAD^+/NADH cycling reaction where NAD^+ and ethanol are converted to NADH and acetaldehyde and NADH and the tetrazolium salt are converted to NAD^+ and formazan. The mixture is incubated and the formazan concentration is measured by light absorption at 550 nm, generally after addition of acid to destroy NADH and stop the cycling reaction.

In the scheme set out above, the sample is referred to as being contacted with the various reagents at the relevant stages of the reaction scheme. However in order to reduce the total number of reagent solutions required, certain reagents may and generally will be added at earlier stages.

Viewed from a further aspect, the invention also provides a kit for a homocysteine assay, said kit comprising:

homocysteine desulphurase, preferably (i) in lyophilized form, the lyophilisate being substantially

- 17 -

free of thiol-containing cryo/lyoprotectants or (ii) in aqueous liquid form further containing a dithiol reducing agent (e.g. DTT, DTE or TCEP) and a proteinaceous or non-proteinaceous stabilizer;

a L-homocyst(e)ine (or L homocysteine precursor) standard, preferably a plurality of standards containing L-HCy or L-homocystine (or a precursor) at a plurality of known concentrations;

a reducing agent, e.g. dithiothreitol, dithioerythiol, TCEP or methyl iodide;

an agent which binds, oxidizes or depotentiates the reducing agent, e.g. an organic disulphide or a dithiol binding agent, preferably a maleimide;

optionally one or more further reagents capable of converting the homocysteine conversion product of HDS into a detectable analyte (e.g. LDH, NADH, ADH, a tetrazolum salt, an oxidizing agent, and an acid);

preferably a pyruvate deactivating agent, e.g. hydrazine, acetoacetate decarboxylase, pyruvate carboxylase, hydrogen peroxide or pyruvate dehydrogenase;

optionally one or more additional reagents capable of removing/inactivating the pyruvate deactivating agent, i.e. catalase; and

optionally a filter capable of removing red blood cells from blood.

The publications mentioned herein are hereby incorporated by reference.

The invention will now be illustrated further by the following non-limiting Examples, with reference to the drawings in which:-

Figure 1 shows the comparison of background signal with and without maleimide and BSA, as a plot of mean OD 550 nm versus time in minutes. Three plots are shown, demonstrating the data obtained from Example 5, (A)♦ no maleimide plus BSA, (B)■ maleimide plus BSA and (C)▲ maleimide without BSA. The experimental conditions are

defined in Example 5;

Figure 2 shows the correlation of results from homocysteine-containing samples from Example 6 read on IMX and by the enzymatic method, as a plot of IMX value versus estimated concentration via enzymatic assay in μM . The experimental conditions are defined in Examples 6, 7 and 8.

Figure 3 shows the results from Example 6, the effect of removal of pyruvate with pyruvate carboxylase, as a plot of OD 550 nm versus concentration of homocysteine in μm . Four plots are shown, \blacklozenge sodium bicarbonate, ATP and pyruvate carboxylase, \blacksquare sodium bicarbonate, ATP without pyruvate carboxylase, \blacktriangle potassium bicarbonate, ATP and pyruvate carboxylase, and X potassium bicarbonate, ATP without pyruvate carboxylase.

Example 1

Assay Reagents

A) Pyruvate and keto acid remover

0.47% Hydrogen peroxide
(Can use 0.47% to 10%)

B) Enzyme Reagent 1

Homocysteine desulphurase	0.02 U/mL
Lactate dehydrogenase	20.8 $\mu\text{g/mL}$
NADH	50 μM
Cryo/lyoprotectant*	0.8 wt %
Phosphate buffer (pH 8.0)	0.1 M
catalase	300 U/ml
Total volume	1.5 mL

* trehalose, gelatin, maltose, dextran, mannitol, Tween 20 or casein

- 19 -

The reagent is in lyophilized form and may be reconstituted with 1.5 mL Ro grade water. It is then stable for 8 hours.

C) Blank Reagent 1

As enzyme reagent 1 but without the homocysteine desulphurase.

D) Reducing reagent

Dithiothreitol 20 mM in aqueous 2.5 mM citric acid, pH 3.0.

E) Reagent 2

190 mM HCl containing 0.55% wt Nonidet P40, 1.5 mM maleimide, and 5.5% ethanol

F) Reagent 3

16 μ M MPMS (1-methoxy-5-methyl-phenazinium methyl sulfate); 144 μ M NBT, 26U/ml ADH, in 963mM Tris buffer pH 7.6.

3.25 mL of 0.1 M phosphate buffer, pH 7.0 (0.026g disodium hydrogen orthophosphate and 0.016g sodium dihydrogen orthophosphate 1-hydrate, in Ro grade water), containing 1% sucrose (maltose or trehalose) (32mg), 23.7 U/ml ADH (alcohol dehydrogenase) and 23.9 U/ml MPMS (1-methoxy-5-methyl-phenazinium methyl sulfate). This is freeze-dried and reconstituted in 13 ml of 144 μ M NBT (nitroblue tetrazolium) in 1.15M Trizma, pH 7.6

- 20 -

G) Calibrator

To make 1 ml of calibrator

L-Homocysteine	0.86 mg
6M HCl	0.50 μ l
Milli-Ro water	9.50 μ l

This is added to 990 μ l of phosphate buffered saline (0.01M phosphate buffer containing 0.0027M KCL, 0.137M NaCl, pH 7.4). This is diluted further with phosphate buffered saline to give stable liquid calibrations of 2.5 μ M, 5 μ M, 10 μ M and 20 μ M, L-homocysteine which corresponds to 5, 10, 20 and 40 μ M L-homocysteine.

H) Stop Solution

6M HCl.

Example 2Assay protocol

Human blood was collected into vacutainer tubes containing citrate. Plasma was separated from cells upon centrifugation at 1000 x g for 10 minutes at 2-8°C.

10 μ l of sample is mixed with 10 μ l of 0.47% Hydrogen peroxide on a microtitre plate and incubated at room temperature for 3 minutes. 25 μ l of Enzyme Reagent 1 is added and incubated for 30 minutes at 37°C. 10 μ l of the same sample is mixed with 10 μ l of 0.47% Hydrogen peroxide and incubated at room temperature for 3 minutes. 25 μ l of Blank Reagent 1 is added and incubated for 30 minutes at 37°C. Following this incubation 85 μ l of Reagent 2 is added to each and after mixing they are incubated a further 3 minutes at room temperature.

- 21 -

Reagent 2 contains the DTT binding agent and the acid destroys the excess NADH. 125 μ l of reagent 3 is added and incubated at 37°C for 15 minutes. Reagent 3 brings the pH of the reaction to pH 7.0 and allows ADH to convert ethanol to acetaldehyde thus generating NADH. The NADH is then converted to NAD⁺ as the colourless tetrazolium salt is converted into the coloured product, aided by the oxidising agent, MPMS. The reaction is stopped by the addition of 15 μ l of 6M HCL and the sample is read at 550nm. The reading obtained for the sample treated with Blank Reagent 1 is subtracted from the reading for the sample treated with Enzyme Reagent 1.

Calibrators are assayed by the same method and a calibration curve is constructed using the delta reading (the reading obtained for the calibrator assayed in the presence of Blank Reagent 1 is subtracted that obtained for the same calibrator assayed in the presence of Enzyme Reagent 1) plotted against the known concentration of the calibrators. The Delta reading obtained for the sample is read from the standard line and a concentration of homocysteine assigned.

Example 3

Background Removal

The assay is performed as in Example 2, with the exception of the pre-treatment of samples with Hydrogen peroxide and the absence of Catalase in Reagent 1 for one set of samples. Table 1 represents samples assayed in the presence and absence of H₂O₂/Catalase. Each sample was assayed four times with Enzyme reagent 1 and four times with Blank Reagent 1. The concentration of homocysteine in each sample was obtained by subtracting the mean reading obtained for each sample assayed with Blank Regent 1 from the individual readings obtained for

each sample assayed in the presence of Enzyme Reagent 1. The results presented in table 1 demonstrate that the background is reduced when samples were assayed in the presence of hydrogen peroxide and Catalase. The reduction in background has improved the precision of the assay by decreasing the percentage CV (coefficient of variance).

Samples	Absence of H ₂ O ₂ /Catalyse			Presence of H ₂ O ₂ /Catalyse		
	Background reading	[HCY] μ M	%CV	Background reading	[HCY] μ M	%CV
1	1.30	3.6	57.5	0.27	8.2	8.9
2	1.24	2.5	63.0	0.24	17.9	5.9
3	1.20	5.1	40.8	0.16	13.5	3.3
4	1.00	7.4	19.7	0.15	12.6	9.4
5	0.82	22.5	11.4	0.22	38.1	1.4
6	1.15	3.9	64.6	0.17	10.8	4.3

[HCY] refers to L-Homocysteine concentration

Table 1 Sample Background and Precision Obtained in the Absence of Hydrogen Peroxide/Catalase

The results presented in Table 1 were also assayed by the Abbott Imx[®] Homocysteine assay. The Homocysteine concentrations obtained for these samples are represented in Table 2 when H₂O₂/Catalase was not present the correlation was found to be $R^2=0.89$ and in the presence of H₂O₂/Catalase the correlation was $R^2=0.99$.

Sample	Homocysteine Concentration (μM)
1	8.3
2	15.3
3	13.8
4	11.4
5	39.1
6	11.2

Table 2 - Homocysteine concentration of samples as determines by Abbot Imx Assay.

Example 4

Assay Performance in comparison to current techniques

The assay was performed as in Example 2. A selection of plasma samples including samples from patients with renal failure, samples from healthy volunteers from two sites collected in either citrated or EDTA vacutainers were assayed in the presence of H_2O_2 /Catalase. Each sample was assayed four times in the presence of Enzyme Reagent 1 and four times in the presence of Blank Reagent 1. The concentration of homocysteine in each sample was obtained by subtracting the mean reading obtained for each sample assayed with Blank Reagent 1 from the individual readings obtained for each sample assayed in the presence of Enzyme Reagent 1. The concentrations obtained were correlated with those obtained in the Abbot Homocysteine IMX assay. The correlation was found to be $R^2=0.96$ and the results are represented in table 3.

Table 3 - Homocysteine Concentrations, %CV of samples treated with H₂O₂ and Catalase

Sample No.	Specific values (obtained in the presence of enzyme reagent 1)				Mean blank (in the presence of blank reagent 1)	Estimated conc. (μM)	%CV	Imx value
	+	+	+	+				
A22	0.291	0.29	0.293	0.284	0.21	10.8	4.7	7.63
A26	0.216	0.214	0.214	0.218	0.138	11	2.5	6.65
A29	0.231	0.217	0.235	0.23	0.137	13.07	8.7	12
A31	0.282	0.28	0.284	0.276	0.168	15.9	2.7	10.97
A32	0.275	0.269	0.281	0.272	0.161	15.9	4.1	11.5
A36	0.279	0.284	0.281	0.272	0.206	10	6.6	7.55
A39	0.298	0.208	0.304	0.296	0.193	15.3	4.6	8.67
A40	0.286	0.286	0.284	0.286	0.189	12.9	1	9.18
A41	0.255	0.263	0.244	0.265	0.154	14.67	9.4	
A42	0.229	0.23	0.222	0.222	0.154	10.16	6.2	6.52
A44	0.247	0.248	0.243	0.243	0.139	13.4	2.6	7.57
A45	0.249	0.254	0.251	0.253	0.173	9.8	3.1	10.9
B47	0.411	0.411	0.423	0.404	0.277	17.9	5.7	18.82
B57	0.53	0.533	0.55	0.569	0.185	55.8	4.9	55.25
B73	0.365	0.375	0.368	0.326	0.186	24.7	6.4	20.4
C8002762	0.516	0.527	0.521	0.527	0.264	40.1	2	42.47
C8002524	0.396	0.413	0.375	0.396	0.221	27.1	8.8	26.48
C8002384	0.429	0.423	0.429	0.426	0.249	27.6	1.6	27.13
C8002610	0.426	0.444	0.445	0.438	0.236	26.8	4.2	27.69
C8002923	0.408	0.391	0.401	0.39	0.193	26.9	4.1	25.83
C8003302	0.257	0.258	0.249	0.253	0.171	11.2	4.81	10.18
C8003316	0.33	0.334	0.353	0.334	0.247	15.1	8.9	12.98
C8003200	0.358	0.356	0.355	0.36	0.174	25.8	1.3	21.56
C8002545	0.448	0.461	0.466	0.472	0.226	33.5	4.5	32.78
C8002369	0.499	0.49	0.498	0.475	0.268	31.6	5.2	30.1
C375262	0.29	0.301	0.307	0.307	0.176	17.88	6.5	15

Sample No.	Specific values				Mean blank (in the presence of blank reagent 1)	Estimated conc. (μ M)	%4.5C V	Imx value
	+	+	+	+				
C485969	0.219	0.229	0.216	0.228	0.145	11.13	8.4	9.48
C357556	0.355	0.338	0.347	0.329	0.221	17.3	9.4	17.4
C515036	0.358	0.341	0.358	0.363	0.234	16.7	7.2	15.61
C513311	0.303	0.303	0.288	0.285	0.164	18.2	6.7	13.81
C483375	0.341	0.351	0.355	0.366	0.185	22.9	5.7	23.52
C489490	0.24	0.231	0.237	0.24	0.124	15.9	3.4	13.34
D190599 25	0.25	0.256	0.255	0.244	0.149	13	5.8	
D190599 32	0.168	0.17	0.169	0.175	0.11	7.4	5.4	9.48
D190599 28	0.217	0.21	0.214	0.212	0.151	7.9	5.6	
D190599 29	0.276	0.28	0.282	0.281	0.147	18.4	1.8	
D190599 30	0.202	0.2	0.2	0.195	0.134	8.2	5.3	
D190599 31	0.197	0.202	0.196	0.194	0.126	11.2	4.6	7.4
E701003	0.248	0.253	0.241	0.243	0.167	9.9	7.5	
E701008	0.359	0.361	0.366	0.359	0.231	17	2.7	11.72
E701005	0.254	0.242	0.254	0.255	0.178	9.4	9.7	6.25
E701006	0.302	0.306	0.301	0.293	0.189	15	5.4	13.04
E701004	0.225	0.231	0.23	0.228	0.146	12.9	3.1	9.06

The correlation between obtained values and values obtained via Imx have been plotted on Figure 2.

Example 5

Comparative assay performance in the presence/absence of BSA and DTT

Background signals for homocysteine assays were determined for a centrifuged but unfiltered plasma sample using the LDH and tetrazolium/formazan system of Examples 1 and 2 under three different assay conditions: (A) the blank enzyme reagent 1 contained BSA as the

- 26 -

cryo/lyoprotectant, and glutamate, alanine aminotransferase, pyridoxyl-5-phosphate and maleimide were not used; (B) the blank enzyme reagent 1 contained BSA as the cryo/lyoprotectant, glutamate, alanine aminotransferase and pyridoxyl-5-phosphate were not used; and (C) the blank enzyme reagent 1 was used in non-lyophilized solution form with no BSA present, and reagent glutamate, alanine aminotransferase and pyridoxyl-5-phosphate were not used.

The background signals for incubation times of 15 to 40 minutes for (A) ♦, (B) ■ and (C) ▲ are shown in Figure 1 of the accompanying drawings.

Example 6

Assay Protocol

The assay was performed as in Example 2, however in place of pre-treatment of sample with Hydrogen peroxide and the inclusion of Catalase in Reagent 1, the sample is pre-treated with Pyruvate Carboxylase.

The sample is mixed with the following combination of reagents: 100 mM trizma base, pH 7.5, 1 mM ATP (adenosine tri-phosphate), 5 mM magnesium chloride, 15 mM sodium or potassium bicarbonate, 0.1 mM acetyl coenzyme A, 0.117 U/ml pyruvate carboxylase and incubated for 90 minutes at 37°C.

The results from one such study are depicted graphically on Figure 3. The signals for varying homocysteine concentration of 0 to 100 μ M are shown.

Example 7Assay Protocol

The assay is performed as in Example 2, however in place of pre-treatment of sample with hydrogen peroxide and the inclusion of catalase in Reagent 1, the plasma or serum sample is filtered through a 30 kD exclusion filter and centrifuged at 10,000 x g for 10 minutes. The assay then proceeds as before. The results showing the reduction in background signal are represented on table 4.

Sample	Background reading before filtration	Background reading after filtration
7	0.18	0.13
8	0.21	0.14

Table 4 background signal levels before and after filtration.

Example 8Assay Protocol

The assay is performed as in Example 2, however in place of pre-treatment of sample with hydrogen peroxide and the inclusion of catalase in Reagent 1, the sample is pre-treated with pyruvate oxidase.

The sample is mixed in a 1:1 ratio with pyruvate oxidase at 15 U/ml, 5 mM magnesium chloride, 0.1 mM thiamine pyrophosphate, 0.1 mM flavin adenine dinucleotide and incubated at 37°C for 30 minutes.

Example 9Removal of Background

The assay is performed as in Example 2, however in addition to pre-treatment of the sample, especially a serum sample, the sample is heat treated at 40-60°C for 15 to 60 minutes. The assay proceeds as before following this step. This additional step removes some of the background left following the removal of Pyruvate and keto acids. The results are represented on table 5.

Sample	Background reading before heat treatment	Background reading after heat treatment
9	0.51	0.26
10	0.34	0.23
11	0.35	0.26

Table 5 background signal levels before and after heat treatment.

Example 10Passive coating method

HDS mixed with 0.1M phosphate buffer containing 0.1M NaCl. 50 μ l to 300 μ l of this solution is added to a microtitre plate, and is incubated for 2 hours. The microtitre plate is then washed 3 times with the same buffer solution and dried in a tunnel drier. To improve stability, an overcoat is added and consists of phosphate buffer (0.1M to 0.5M) containing sucrose (1-10%).

Assay using immobilized HDS

1mM DTT is added to the sample, calibrator or control in order to cleave homocysteine from binding partners. The treated sample is then added to the plate, and incubated for 3 minutes. The plate is then washed with phosphate buffer.

The assay is performed as in Example 2, however, Reagent 1 does not contain HDS as it is bound to the plate.

Example 11

Assay Protocol

The assay is performed as in Example 2, however in place of pre-treatment of sample with hydrogen peroxide and the inclusion of catalase in Reagent 1, the plasma or serum sample is pretreated with 20.8 $\mu\text{g/ml}$ LDH and 1 mM NADH and incubated for 30 minutes at 37°C. The NAD⁺ generated can be destroyed by treatment with Nitrous acid or converted into NADH using 100 U/ml ADH and 5% ethanol.

The ADH can be immobilised on a Sepharose support before it is mixed with the sample and therefore removed from the plasma or serum by centrifugation (2 min. at 2000x g). (Alternatively if ADH is not attached to a Sepharose support its excess activity can be removed by an inhibitor (e.g. tetramethylthiuram disulfide an inhibitor of yeast ADH.))

Results obtained are represented on table 6, using immobilised ADH. The reduction in background signal is represented kinetically with a reduction in slope.

Sample	Slope (mAU/min at 550 nm)
In the presence of immobilised ADH	14
In the presence of immobilised ADH	88

Table 6 - The effect of LDH pre-treatment and NAD⁺ removal on background

Claims

1. An assay for homocysteine which comprises contacting a biological fluid sample with a reducing agent and subsequently with homocysteine desulphurase, characterised in that said sample is contacted with an agent which binds, oxidizes or depotentiates said reducing agent after being contacted with said homocysteine desulphurase.
2. A homocysteine assay which comprises contacting a biological fluid sample with a liquid reagent containing a homocysteine converting enzyme, wherein said reagent is produced by adding an aqueous liquid to a lyophilisate containing said enzyme and a cryo/lyoprotectant, characterised in that said lyophilisate is substantially free of thiol-containing cryo/lyoprotectants.
3. A homocysteine assay which comprises contacting a biological fluid sample with a liquid reagent containing homocysteine desulphurase, wherein said liquid reagent is an aqueous liquid containing homocysteine desulphurase, a thiol-reducing reagent, and a proteinaceous or non-proteinaceous stabilizer.
4. A homocysteine assay which comprises contacting a biological fluid sample with a homocysteine converting enzyme, characterised in that before contact with said enzyme said sample is treated with an agent which serves to deactivate pyruvates, e.g. by immobilizing, binding or converting pyruvates.
5. A homocysteine assay which comprises contacting a biological fluid sample with an immobilized homocysteine converting enzyme, wherein said biological fluid sample contacts the immobilized enzyme under such time and

- 32 -

conditions to allow the homocysteine in the sample to bind to said enzyme, characterised in that the biological fluid sample is then removed from the assay.

6. A homocysteine assay which comprises contacting a biological fluid sample with a homocysteine converting enzyme, characterised in that before contact with said enzyme said sample is filtered through an exclusion filter and centrifuged in order to remove pyruvates.

7. An assay as claimed in at least two of claims 1 to 6.

8. An assay as claimed in at least three of claims 1 to 6.

9. An assay as claimed in at least four of claims 1 to 6.

10. An assay as claimed in claims 1, 2 and 4.

11. An assay as claimed in claims 1, 3 and 4.

12. An assay as claimed in claim 4 wherein the agent which serves to deactivate pyruvates is hydrogen peroxide.

13. An assay as claimed in claim 12 wherein the hydrogen peroxide is neutralised prior to contacting the sample with said homocysteine converting enzyme using catalase.

14. An assay as claimed in any one of claims 4, 12 or 13 wherein after the sample is treated with the said agent, the sample is heated at 40-60°C for 15 to 60 minutes prior to contacting with said homocysteine converting enzyme.

- 33 -

15. An assay as claimed in claim 4 wherein the agent which serves to deactivate pyruvates is pyruvate carboxylase.

16. An assay as claimed in claim 4 wherein the agent which serves to deactivate pyruvates is pyruvate oxidase.

17. An assay as claimed in claim 4 wherein the agent which serves to deactivate pyruvates is lactate dehydrogenase.

18. An assay as claimed in claim 6 wherein the sample is filtered with a 30 kD exclusion filter.

19. An assay as claimed in any one of claims 1 to 18 wherein said homocysteine converting enzyme is HDS and wherein a NAD^+/NADH cycling reaction is used to generate a coloured compound the concentration of which may be correlated to the concentration of homocysteine in the initial biological fluid sample.

20. A kit for a homocysteine assay, said kit comprising:

homocysteine desulphurase, preferably (i) in lyophilized form, the lyophilisate being substantially free of thiol-containing cryo/ lyoprotectants or (ii) in aqueous liquid form further containing a dithiol reducing agent (e.g. DTT, DTE or TCEP) and a proteinaceous or non-proteinaceous stabilizer;

a homocyst(e)ine standard, preferably a plurality of standards containing HCy or homocystine at a plurality of known concentrations;

a reducing agent, e.g. dithiothreitol, dithioerythiol, TCEP or methyl iodide;

an agent which binds, oxidizes or depotentiates the reducing agent, e.g. an organic disulphide or a dithiol

- 34 -

binding agent, preferably a maleimide;

optionally one or more further reagents capable of converting the homocysteine conversion product of homocysteine desulphurase into a detectable analyte;

preferably a pyruvate deactivating agent, e.g. hydrazine, acetoacetate decarboxylase, pyruvate carboxylase, hydrogen peroxide or pyruvate dehydrogenase;

optionally a filter for removing pyruvate, i.e. a molecular sieve; and

optionally a filter capable of removing red blood cells from blood.

1/3

Comparison of Background With and Without Maleimide and BSA

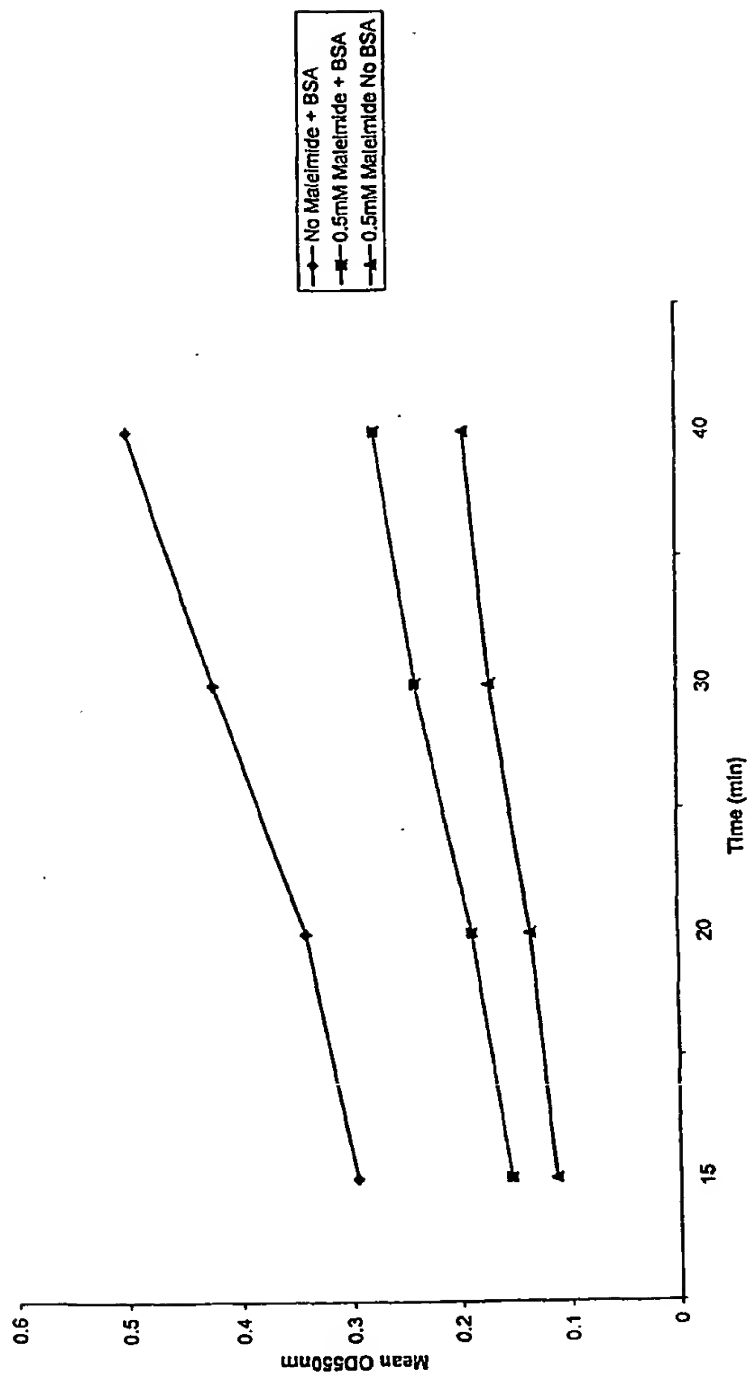


FIGURE 1

2/3

Comparison of Homocysteine Concentration values obtained on samples read on IMX and enzymatic assay.

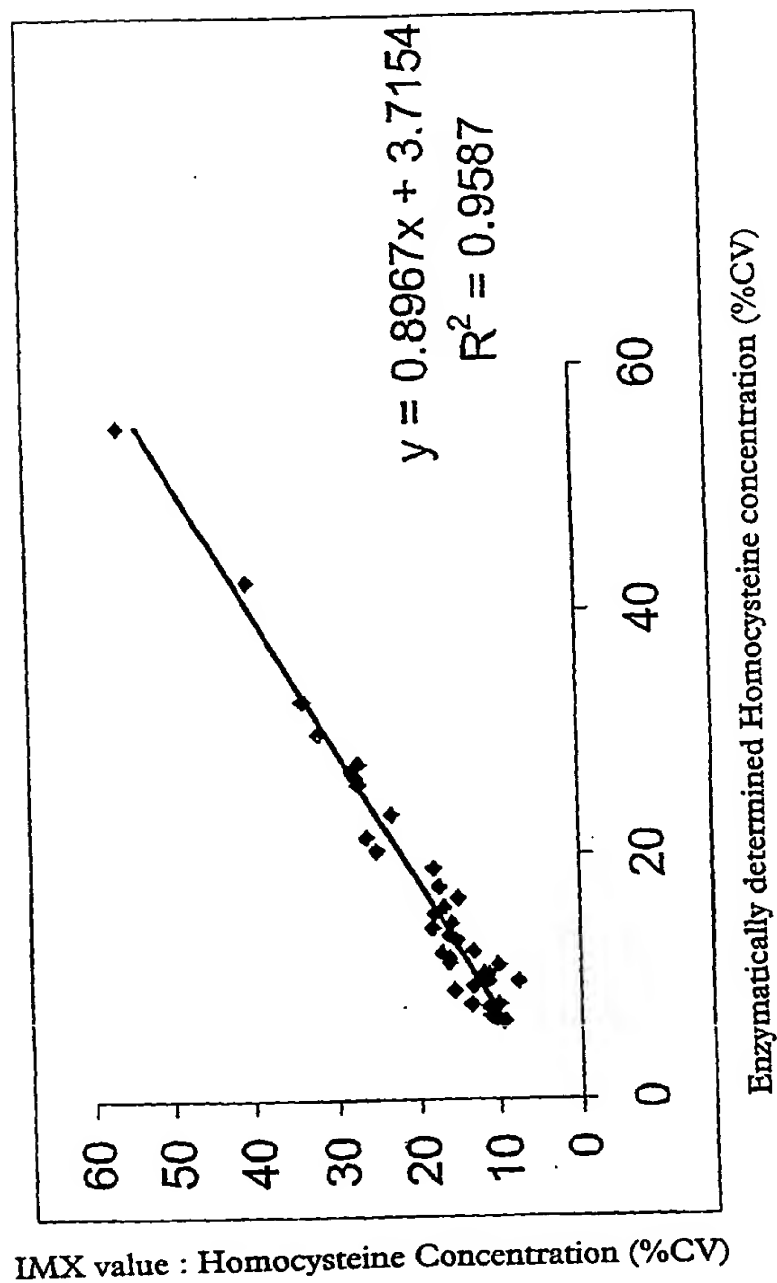


FIGURE 2

3/3

Pyruvate carboxylase activity on pyruvic acid

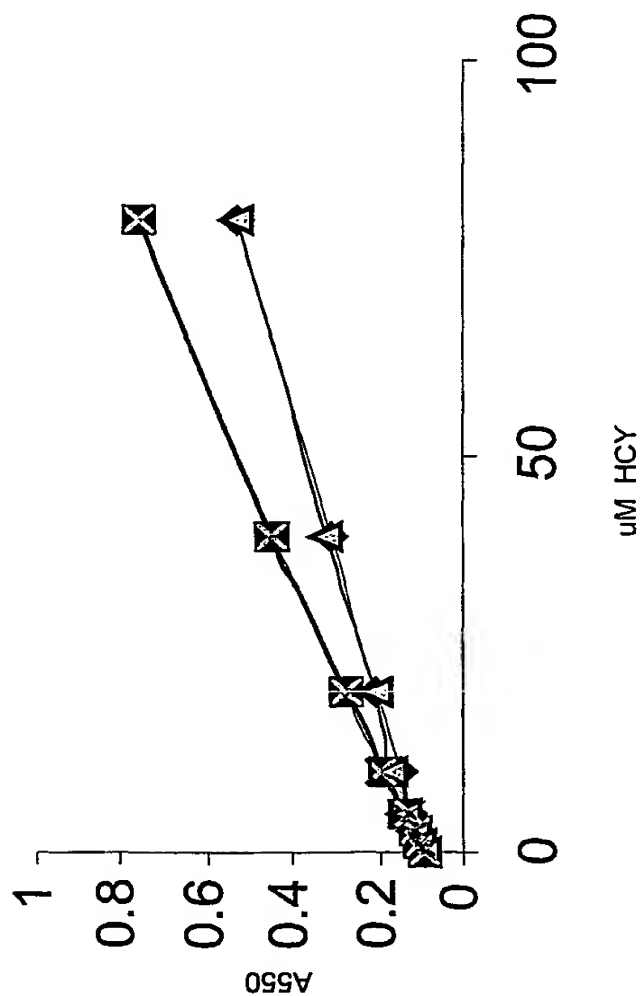


FIGURE 3

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 October 2001 (18.10.2001)

PCT

(10) International Publication Number
WO 01/77670 A3

- (51) International Patent Classification⁷: **C12Q 1/527**
- (21) International Application Number: **PCT/GB01/01615**
- (22) International Filing Date: **10 April 2001 (10.04.2001)**
- (25) Filing Language: **English**
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- (30) Priority Data:
0008784.1 10 April 2000 (10.04.2000) GB
- (71) Applicant (for all designated States except US):
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- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **CONNOLLY, Caroline** [GB/GB]; Axis-Shield Plc, Luna Place, The Technology Park, Dundee DD2 1XA (GB). **BRADY, Jeff** [GB/GB]; Axis-Shield Plc, Luna Park, The Technology Park, Dundee DD2 1XA (GB).
- (74) Agents: **COCKBAIN, Julian** et al.; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).
- (81) Designated States (national): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report
- (88) Date of publication of the international search report:
16 May 2002
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **HOMOCYSTEINE ASSAY**

(57) Abstract: The present invention provides an improved method of assessing/quantifying the amount of homocysteine in a body fluid sample via an enzymatic assay which comprises reducing background signal by treatment with one of the following: a reducing agent, a pyruvate deactivating agent, heat treatment, or by lyophilising or immobilizing the homocysteine converting enzyme.

WO 01/77670 A3

INTERNATIONAL SEARCH REPORT

Application No
PCT/GB 01/01615

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/527

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 998 191 A (LENZ MARTIN ET AL) 7 December 1999 (1999-12-07) cited in the application examples	1-6,20
A	WO 00 00821 A (ULLMAN EDWIN F) 6 January 2000 (2000-01-06) abstract	1-6,20
A	WO 98 59242 A (NEXSTAR PHARMACEUTICALS INC) 30 December 1998 (1998-12-30) abstract	1-6,20
A	EP 0 726 322 A (AXIS BIOCHEMICALS AS) 14 August 1996 (1996-08-14) abstract	1-6,20

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *S* document member of the same patent family

Date of the actual completion of the international search

18 January 2002

Date of mailing of the international search report

28/01/2002

Name and mailing address of the ISA

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Authorized officer

Moreno, C

INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No

PCT/GB 01/01615

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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			US 5827645 A	27-10-1998

1

11

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

To:

FRANK B. DEHN & CO.
Attn. Cockbain, Julian
179 Queen Victoria Street
London EC4V 4EL
UNITED KINGDOM

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

72334/001
28 JAN 2002

RECEIVED

ANSO

Date of mailing
(day/month/year)

28/01/2002

Applicant's or agent's file reference

44.7.72334/001

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No.

PCT/GB 01/01615

International filing date
(day/month/year)

10/04/2001

Applicant

AXIS-SHIELD PLC et al.

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Geertruida Groeneveld-Van der Spek

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 44.7.72334/001	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/GB 01/ 01615	International filing date (day/month/year) 10/04/2001	(Earliest) Priority Date (day/month/year) 10/04/2000
Applicant AXIS-SHIELD PLC et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.



It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.



the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :



contained in the international application in written form.



filed together with the international application in computer readable form.



furnished subsequently to this Authority in written form.



furnished subsequently to this Authority in computer readable form.



the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.



the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐

Certain claims were found unsearchable (See Box I).

3. ☐

Unity of invention is lacking (see Box II).

4. With regard to the title,



the text is approved as submitted by the applicant.



the text has been established by this Authority to read as follows:

5. With regard to the abstract,



the text is approved as submitted by the applicant.



the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.



as suggested by the applicant.



because the applicant failed to suggest a figure.



because this figure better characterizes the invention.



None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No

GB 01/01615

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/527

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 998 191 A (LENZ MARTIN ET AL) 7 December 1999 (1999-12-07) cited in the application examples	1-6, 20
A	WO 00 00821 A (ULLMAN EDWIN F) 6 January 2000 (2000-01-06) abstract	1-6, 20
A	WO 98 59242 A (NEXSTAR PHARMACEUTICALS INC) 30 December 1998 (1998-12-30) abstract	1-6, 20
A	EP 0 726 322 A (AXIS BIOCHEMICALS AS) 14 August 1996 (1996-08-14) abstract	1-6, 20

☐ Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

18 January 2002

Date of mailing of the international search report

28/01/2002

Name and mailing address of the ISA

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Authorized officer

Moreno, C

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

GB 01/01615

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